

Persistence of Japanese Encephalitis Virus Is Associated with Abnormal Expression

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Persistent infection with Japanese encephalitis virus (JEV) was established in murine neuroblastoma N18 cells, and the persistency has been maintained in cell culture for over 6 months. From the persistently infected cells, a clone named C2-2 was selected and expanded to form a stable cell line. The vast majority of C2-2 cells showed viral protein staining by immunofluorescence and continuously produced low levels of virus (10^3 to 10^4 PFU/ml) without marked cytopathic effects or cyclic variations. In addition to the wild-type viral proteins, truncated forms of the viral nonstructural protein 1 (NS1) as well as its derivative NS1' were produced in C2-2 cells. Both truncated NS1 and NS1' contain deletions at their N-termini; however, the analyses by RT-PCR and direct sequencing of the viral RNA failed to detect any truncations or mutations within the NS1 region, suggesting that NS1 truncation was a result of a unique posttranslational proteolytic cleavage of NS1 in the persistently infected cells. Similar but not identical truncation of NS1 was also observed in two other persistently infected cell lines established in Vero and DBT (murine astrocytoma) cells. However, viruses released from C2-2 cells did not produce truncated NS1 upon infection of N18 cells, suggesting that NS1 truncations were the result of virus-cell interaction in persistently infected cells. These data indicate a strong association between abnormal NS1 expression and JEV persistency. A probable involvement of dysfunctional NS1 in the establishment and/or maintenance of JEV persistency in tissue culture is discussed. © 1996 Academic Press, Inc.

INTRODUCTION

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* and causes acute encephalitis in humans with a high mortality rate (Burke and Leake, 1988; Vaughn and Hoke, 1992). Similar to the majority of viruses in this family, JEV is arthropod-borne and transmitted to human through persistently infected mosquito vectors. In the natural life cycle of JEV, human is the dead-end host, while other vertebrates, such as swine, serve as an amplifying reservoir for the maintenance of JEV (Chambers *et al.*, 1990).

The JEV genome is a single-stranded, positive-sense RNA of approximately 11 kb and contains an open reading frame of more than 10 kb encoding a polyprotein (reviewed in Chambers *et al.*, 1990). In the infected cells, the polyprotein is proteolytically cleaved by cellular and viral proteases into more than 10 viral proteins. The structural proteins, including capsid (C), precursor of membrane (prM), and envelope (E), are encoded in the 5' quarter of the genome, and the nonstructural proteins, designated NS1 through NS5, are encoded in the remain-

der. Characteristically, the order of flaviviral proteins is 5'-C-prM(M)-E-NS1-NS2A2B-NS3-NS4A4B-NS5-3'.

The nonstructural proteins in JEV-infected cells comprise a glycosylated NS1; two hydrophilic proteins, NS3 and NS5; and four small hydrophobic proteins, NS2A, NS2B, NS4A, and NS4B, associated with the cellular membrane (Hashimoto *et al.*, 1990; Nitayaphan *et al.*, 1990; Sumiyoshi *et al.*, 1987). NS1 has a predicted molecular weight of 42 kDa, with two N-linked carbohydrate chains at positions 105 and 182; however, the actual size of NS1 in JEV-infected cells is approximately 48 kDa. The proteolytic cleavage of E-NS1 is followed by the translocation of NS1 into the lumen of the endoplasmic reticulum (ER), and the cleavage of NS1-2A is thought to take place in the lumen of vesicular compartments (Chambers *et al.*, 1990). An additional NS1-2A-related protein (named as NS1') of 56 kDa is often observed in JEV-infected cells (Mason, 1989) and is presumably generated by a protease which recognizes an alternative cleavage site within NS2A (Jan *et al.*, 1995). It has been suggested that NS1 may play a role in virion maturation, since NS1 is coretained with the immature forms of the structural glycoprotein E in the ER of JEV-infected cells (Fan and Mason, 1990). Nonetheless, the exact biological functions of NS1 and NS1' in JEV replication remain unclear.

JEV is usually cytolytic for susceptible cells; yet, persis-

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tent infection has been demonstrated in cell cultures (Schmaljohn and Blair, 1977, 1979; Shah and Gadkari, 1987) as well as in a mouse model (Mathur *et al.*, 1986). Moreover, in the human, latent infection of mononuclear cells in the peripheral blood from JEV-infected patients has been documented (Sharma *et al.*, 1991); viral persistency in the human nervous system has been shown in approximately 5% of JEV-associated encephalitis (Ravi *et al.*, 1993), suggesting that it might be responsible for the neural sequelae after acute infection phase. The precise mechanism for JEV persistency *in vitro* and *in vivo* has not been fully understood. One of the possible mechanisms that have been suggested for other flaviviruses to persist in cell cultures is the production of defective interfering (DI) particles (Brinton, 1986; Poidinger *et al.*, 1991; Schmaljohn and Blair, 1977). However, the production of DI virus has not been universally observed. The present study describes the establishment of persistent JEV infection in murine neuroblastoma N18 cells and demonstrates a novel mechanism of persistent infection involving the synthesis of an abnormal NS1 protein. To the best of our knowledge, this is the first report of persistent JEV infection in immortalized neuronal cells. This system provides an *in vitro* model for studying the molecular process of persistent JEV infection in neurons. Determination of viral and cellular factors involved in the establishment and maintenance of JEV persistency will aid our understanding of viral replication and chronic disease state of JEV infection.

MATERIALS AND METHODS

Cell lines and viruses

The cell lines used to establish JEV persistency in this study were as follows: N18, a mouse neuroblastoma cell line (Amano *et al.*, 1972) (a kind gift from Dr. D. E. Griffin, Johns Hopkins University, Baltimore MD); Vero, a monkey kidney cell line; and DBT, a mouse astrocytoma cell line (Hirano *et al.*, 1974). C2-2 was a clone selected by limiting dilution (see below) from the persistently JEV-infected N18 cells. All cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS). A JEV strain RP9, a plaque-purified virus from a Taiwan local strain NT-109 which was originally isolated from infected mosquito *Cx. tritaeniorhynchus*, was used throughout this study. The propagation of virus was carried out in BHK-21 cells using RPMI 1640 medium containing 2% FCS. Virus titers were determined by plaque-forming assay on BHK-21 cells.

Cloning of persistently infected cells

Limiting dilution was used to clone persistently infected cells. Briefly, the bulk of persistently JEV-infected N18 cells was trypsinized and washed once with complete RPMI 1640 medium. Cell suspensions were

counted, diluted with medium, and distributed into a 96-well microtiter plate. Each well, which contained an average of one cell, was microscopically observed daily for the presence of cell colonies. Once they became confluent in wells, cells were trypsinized and transferred to new 25-cm tissue culture flasks for further growth.

Generation and characterization of monoclonal antibodies

Six- to eight-week-old BALB/c mice were immunized intraperitoneally with JEV-infected suckling mouse brain suspensions mixed with an equal volume of Freund's complete adjuvant for first inoculation, and incomplete Freund's adjuvant was used for subsequent boosting. JEV-specific antisera were obtained from the mice after three consecutive challenges. For generation of monoclonal antibodies, splenocytes were fused with NS-1 myeloma cells and selected as described by Kohler and Milstein (1976). The hybridoma secreting specific antibodies were identified by ELISA as described by Heinz *et al.* (1983) and by immunoprecipitation assays using JEV-infected BHK-21 cell lysates as antigen source. Single-cell clones were generated by limiting dilution and the cells were injected into incomplete Freund's adjuvant-primed BALB/c mice for ascitic fluid production.

The specificity of five monoclonal antibodies, D2/8-1, Na39-3.1, Na41-2, JE7/45-2, and Na81-2.1, against JEV NS1 used in this study was further confirmed by Western blotting and immunoprecipitation using antigen source derived from the lysate of BHK-21 cells expressing NS1 by a recombinant plasmid, pJNS1/cDNA3, under CMV promoter control (unpublished data). All five antibodies recognized native forms of NS1, but when the lysate was boiled in the presence of 2-mercaptoethanol prior to SDS-PAGE, only antibody D2/8-1 detected denatured forms of NS1 (data not shown). These data indicated that D2/8-1 recognized a linear epitope of NS1, and that the remaining antibodies recognized conformational epitopes on NS1. The epitope location recognized by D2/8-1 was characterized by several NS1 deletion mutants expressed in BHK-21 cells, and the result showed that the epitope was located in the N-terminal one-third of NS1 (data not shown). Using synthetic peptides for fine mapping, the epitope of D2/8-1 was mapped to a 16-mer peptide whose sequence was TELRYSWKTWGKAKM (data not shown); this peptide contained a consensus linear sequence [WK(A/T)WGK] present in the NS1 of JEV, Kunjin virus, and dengue virus (Falconar and Young, 1991; Falconar *et al.*, 1994).

Indirect immunofluorescence staining

Cells were fixed by acetone/methanol (1/1) solution for 5 min, stained with JEV-specific polyclonal or monoclonal antibodies at room temperature for 1 hr. The dilutions of antibodies used in this assay were 1:500 or 1:1000. After

washing with phosphate-buffered saline (PBS), the cells were reacted with goat anti-mouse fluorescein-conjugated secondary antibody (Cappel) and examined under a Leitz fluorescent microscope.

Cell labeling and immunoprecipitation

Cell monolayers were starved with methionine- and cysteine-free RPMI 1640 for 1 hr and labeled with 50 μCi of ^{35}S Pro-Mix (Amersham) per 35-mm dish for 4 hr at 37°. The cells were rinsed and lysed with lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing a cocktail of protease inhibitors, 20 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride, 2 $\mu\text{g/ml}$ leupeptin, and 2 $\mu\text{g/ml}$ aprotinin. Aliquots of cell lysates were mixed with polyclonal or monoclonal antibodies captured on *Staphylococcal* protein A-coated Sepharose (Pharmacia) for 1 hr at room temperature. The immune complexes were washed with RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate), analyzed by 10% SDS-polyacrylamide gel electrophoresis, and fluorographed at -70°.

Western immunoblot analysis

Cell lysates were mixed with an equal volume of the sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2% SDS, 0.1% bromophenol blue, 10% glycerol) lacking 2-mercaptoethanol with or without boiling, separated by SDS-PAGE, and transferred to nitrocellulose membrane (Hybond-C Super; Amersham). The non-specific antibody-binding sites were blocked with 5% skimmed milk in PBS, and the filters were incubated with monoclonal anti-JEV NS1 antibodies. The blot was then treated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Cappel) and developed with 4-chloro-1-naphthol.

RNA preparation and RT-PCR reaction

Viruses harvested from the media of the JEV-infected cell cultures were incubated with lysis buffer (4 M guanidine thiocyanate, 50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% sarcosyl) for 15 min at 4° and extracted twice with an equal volume of acid phenol/chloroform (Chomczynski and Sacchi, 1987). RNA in the aqueous phase was precipitated by isopropanol. Four different primers were used in the amplification reaction. Primers a [5'-GCG-ATCCAGACACTGGATGTGCCA-3', (+) sense], b [5'-GCGGATCCTAAGCATCAACCTGTGA-3', (-) sense], c [5'-GCATTCTCTTTGCCCGGAATTGGC-3', (+) sense], and d [5'-GGAAGGGGAGACAAGCAGATCAACC-3', (-) sense] correspond to nucleotides 2478-2501, 3510-3534, 2133-2157, and 2839-2863 of the JEV cDNA, respectively. Using primer b or d, 5 μg of purified RNA was transcribed into first-strand cDNA at 42° for 1 hr in 20 μl of reaction buffer containing 200 units of Moloney murine

leukemia viral reverse transcriptase (Bethesda Research Laboratories), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT, and 1 mM each dATP, dGTP, dCTP, and dTTP. Using primer sets a and b or c and d, 2 μl of the resulting first-strand cDNA sample was PCR-amplified in 100 μl of reaction mixture containing 5 units of *Taq* polymerase (Perkin-Elmer); 2 mM MgCl_2 ; 1 mM each dATP, dGTP, dCTP, and dTTP; 20 mM Tris-HCl (pH 8.4); 50 mM KCl. Thirty-five cycles of amplification were performed by using a PCR thermocycler (AG 9600 Thermal Station; AcuGen System). Each cycle consisted of a denaturation step at 94° for 30 sec followed by a primer-annealing step at 55° for 30 sec and a primer extension step at 72° for 30 sec, except for a longer denaturation step (5 min) and a longer primer extension step (10 min) at the first and last cycle.

DNA sequencing

PCR products were purified from agarose gel by Qiaquick gel extraction kit (Qiagen) and sequenced with Sequenase PCR product sequencing kit (United States Biochemical) following the manufacturer's instructions based on the Sanger dideoxynucleotide method (Sanger *et al.*, 1977).

RESULTS

Establishment of persistent JEV infection in mouse neuroblastoma N18 cells

A mouse neuroblastoma cell line N18 was used to establish a persistent JEV infection in cell culture. Like primary infection of BHK-21 cells, JEV is cytolitic to most N18 cells and the virus yield obtained from this primary infection was approximately 1×10^7 PFU/ml. N18 cells were first infected with a plaque-purified JEV strain RP9 at a multiplicity of infection (m.o.i.) of 10. At 30 hr postinfection (p.i.), severe cytopathic effects (CPE) in infected cells were observed, and more than 90% of the cells were lysed by 40 hr p.i. The remaining cells from primary JEV infection were again infected with JEV at m.o.i. 10 for further selection of surviving cells. After three consecutive cycles of selection, surviving cells were maintained and grown into a stable cell population without apparent CPE. The growth rate of this cell population was about 30% slower than the normal N18 cells. To determine whether this was a persistent JEV infection in N18 cells, indirect immunofluorescence assay (IFA) using virus-specific antibodies was carried out to detect viral antigens expressed in these cells; about 90% of the cells showed microscopically strong positive staining in the cytoplasm (data not shown). In addition, a virus titer of about 10^3 to 10^4 PFU/ml was continuously detected in the culture media from bulk cells during the 6-month period. These results indicated that these cells had been persistently infected with JEV.

TABLE 1

Properties of Cell Clones Isolated from JEV-Infected N18 Bulk Cells

Clone	Without SI ^a				With SI		
	Virus titer (PFU/ml)	Expression of viral proteins		RT-PCR	Virus titer (PFU/ml)	Expression of viral proteins	
		IFA	RIP			IFA	RIP
C1-1	0	–	–	No ^b	9.6×10^5	+	+
C1-2	0	–	–	No	7.7×10^5	+	+
C2-1	0	–	–	No	3.3×10^5	+	+
C2-2	2.1×10^3	+	+	+	2.1×10^3	+	+
C2-3	0	–	–	No	3.1×10^5	+	+

^a SI, superinfection with JEV.

^b No, no PCR products detected.

In an attempt to obtain persistently JEV-infected cell clones, the bulk cells were cloned by limiting dilution as described under Materials and Methods. Among 31 cell clones established by such a manner, 5 clones were randomly chosen for further studies, and some of their characteristics are shown in Table 1. During 5-month passages, only 1 clone, C2-2, continued to release a low titer of virus into medium, and nearly 100% of cells in this clone showed positive IFA staining with antibodies against viral proteins (Table 1). However, as analyzed by infectious center assay, only 1% of C2-2 cells were capable of shedding infectious virus. The reason for the discrepancy between the results of IFA and infectious center assay for this clone was unclear (see below under Discussion). The remaining clones had apparently been cured of viral infection during passages, as evidenced by negative IFA staining and lack of viable virus production. The susceptibility of these cell clones to superinfection by homologous viruses was examined (Table 1). After superinfection with JEV-RP9 at m.o.i. 50, all 5 clones showed positive IFA staining; however, all but C2-2 showed severe CPE at 30 hr p.i. and were lysed by 40 hr p.i.; in addition, all of the clones except C2-2 released infectious virus to a titer of about 10^5 PFU/ml into the medium (Table 1), indicating that they were susceptible to homologous superinfection to almost the same extent as primary infection in N18 cells, although the virus yield was slightly lower than that from primary infection of N18 cells (about 5×10^7 PFU/ml). By contrast, the C2-2 clone was resistant to homologous superinfection since no CPE and no changes in virus titers were observed after superinfection (Table 1).

To determine whether all the other clones were indeed "cured" of viral infection, RT-PCR was performed to detect the possible existence of viral genome in four viral antigen-negative clones, C1-1, C1-2, C2-1, and C2-3 (Table 1). The result showed that none of these clones contained JEV RNA; in contrast, JEV RNA was detected in

the C2-2 clone. Therefore, the antigen-negative clones were indeed cured of viral infection during passages.

The susceptibility of C2-2 cells to infection by a heterologous virus, Sindbis virus, which is a prototype alphavirus, was investigated. Severe CPE and cell death were observed at 12 hr p.i. in C2-2 cells and at 24 hr p.i. in the parental N18 cells, indicating that C2-2 cells, similar to N18 cells, were susceptible to heterologous viral infection. Together, these data, including the continuous release of infectious virus, the presence of viral antigens and RNA genomes in the cells, and the susceptibility to heterologous but not homologous viral superinfection, clearly indicate that C2-2 is a *bona fide* persistently JEV-infected cell clone.

Comparison of viral protein expression between primary and persistent infection

To study the expression profiles of viral protein in persistently infected cells, [³⁵S]methionine-labeled cell lysates from primary infection of N18 cells and from persistent infection of C2-2 were precipitated by JEV-specific antibodies and separated by SDS-PAGE (Fig. 1). When using monoclonal antibodies specific for either E (Fig. 1, lanes 3 and 4) or NS3 (Fig. 1, lanes 7 and 8), the patterns of viral antigen expressions were similar between primary and persistent infections, although the amounts of proteins varied. It was not clear why there was slightly more NS3 precipitated in the C2-2 cells (Fig. 1, lane 8) than in the primarily infected N18 cells (lane 7). In contrast, when JEV-specific polyclonal antiserum (Fig. 1, lanes 1 and 2) or monoclonal antibodies specific for NS1 (Fig. 1, lanes 5 and 6) were used, two additional proteins with faster mobility, p45 and p39, were observed in persistently infected C2-2 cells (lanes 2 and 6), but not in primarily infected N18 cells (lanes 1 and 5).

To determine whether this difference in NS1 protein pattern could also be seen between the C2-2 and the viral antigen-negative cell clones described in Table 1,

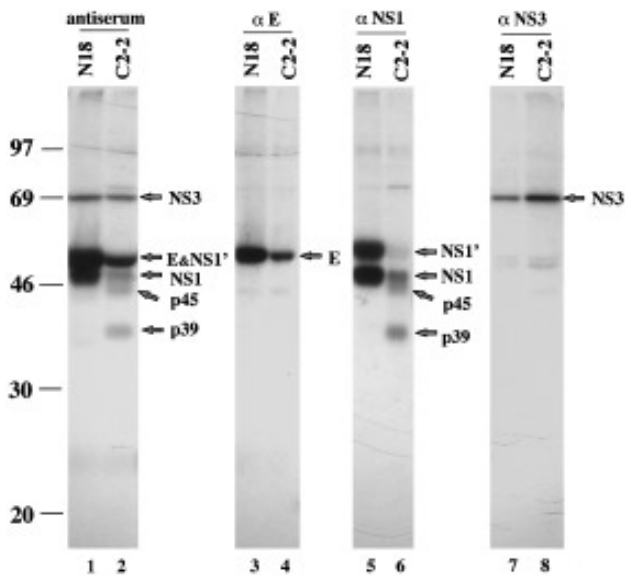


FIG. 1. Comparison of viral antigens expressed between the primarily JEV-infected N18 cells and the persistently infected clone C2-2. [35 S]-Methionine-labeled lysates from N18 and C2-2 cells were immunoprecipitated either with polyclonal antiserum specific for JEV (antiserum, lanes 1 and 2) or with monoclonal antibodies specific for E (α E, lanes 3 and 4), NS1 (α NS1, lanes 5 and 6), or NS3 (α NS3, lanes 7 and 8). The positions of each viral protein in 10% SDS-PAGE are indicated by arrows. Note that two additional proteins, p45 and p39, were found in C2-2 cell lysates when precipitated by polyclonal antiserum (lane 2) and by anti-NS1 monoclonal antibodies (lane 6).

we carried out immunoprecipitation of NS1 in the six clones after superinfection with JEV (Fig. 2). All four antigen-negative clones expressed normal NS1 and NS1' (Fig. 2, lanes 1 to 3 and 5), whereas only C2-2 (Fig. 2, lane 4) produced the two additional abnormal proteins,

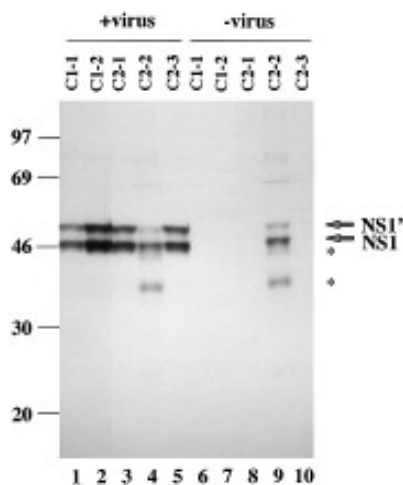


FIG. 2. Expression patterns of NS1 with or without JEV superinfection from five clones derived from the persistently infected N18 bulk cells. Five clones, with (lanes 1 to 5) or without JEV superinfection (lanes 6 to 10), were radioisotope-labeled, and the cell lysates were immunoprecipitated by anti-NS1 monoclonal antibodies. The positions of p45 and p39 are marked by asterisks. Numbers are the molecular weights in kDa indicated on the left.

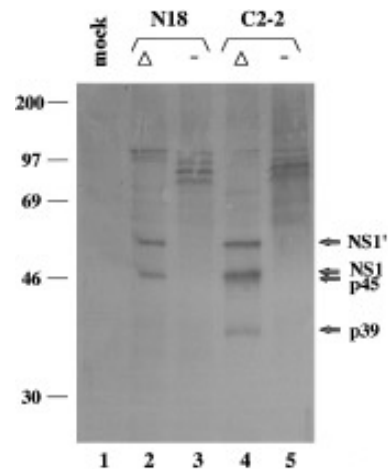


FIG. 3. Immunoblot analysis of NS1 and its derivatives from JEV primary and persistent infections. Cell lysates from mock infection of N18 (lane 1), from primary infection of N18 (lanes 2 and 3), and from persistent infection of C2-2 cells (lanes 4 and 5) were separated by 10% SDS-PAGE and immunoblotted with anti-NS1 monoclonal antibody JE7/45-2. Protein samples were treated either with boiling (Δ , lanes 2 and 4) or without boiling ($-$, lanes 3 and 5). Monomeric NS1, NS1', p45, and p39 are marked by arrows. Molecular weight markers in kDa are on the left.

p45 and p39. As expected, only C2-2 cells (Fig. 2, lane 9) and not the other clones (Fig. 2, lanes 6 to 8 and 10), expressed NS1 protein in the absence of JEV superinfection, consistent with the result of immunofluorescence described in Table 1. These results indicate that the abnormal NS1 expression in C2-2 cells is a property associated with persistently infected viral genome.

To determine whether the truncation of the NS1 was an inherent property of the viruses released from the persistently infected C2-2 cells, fresh N18 cells were infected with these viruses and the pattern of NS1 proteins in the infected cells was analyzed. To eliminate the possible influence from DI particles, the released viruses from C2-2 were first amplified once in BHK-21 cells at m.o.i. 0.01. Similar to the primary JEV infections, the primary infection of N18 cells with the viruses released from C2-2 resulted in lytic infection after 30-hr incubation, and no abnormal forms of NS1 proteins were detected in the infected cells (data not shown). These results suggested that the aberrant NS1 expression was not an inherent property of the viruses released from C2-2 cells, but most likely the result of virus-cell interaction associated with persistent infection.

Characterization of the truncated NS1 proteins from persistently infected C2-2 cells

To determine if the faster migrating proteins, p45 and p39, detected in the persistently infected C2-2 cells are truncated forms of NS1 or cellular proteins associated with NS1, we carried out immunoblotting analysis using a monoclonal antibody, JE7/45-2, specific for NS1. As shown in Fig. 3, when protein samples were heat-dena-

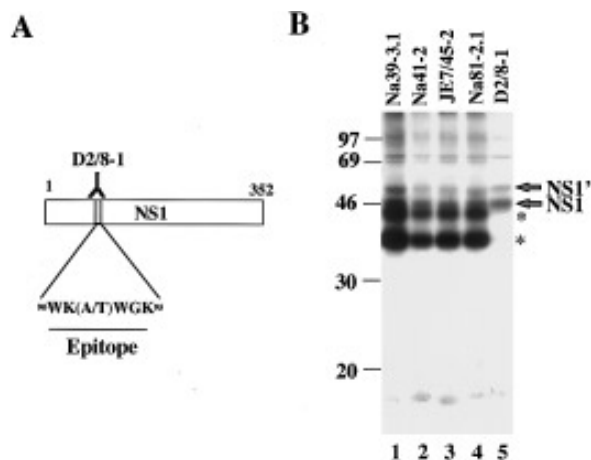


FIG. 4. Localization of the truncation of NS1 and its derivatives to their N-termini in C2-2 cells by immunoprecipitation using anti-NS1 monoclonal antibodies. (A) Schematic diagram of monoclonal antibody D2/8-1 which binds to the N-terminal one-third of NS1 and recognizes a common linear epitope ($^{90}\text{WK(A/T)WGK}^{95}$) (see Materials and Methods). (B) ^{35}S -labeled lysates from C2-2 cells were precipitated by five anti-NS1 monoclonal antibodies, Na39-3.1, Na41-2, JE7/45-2, Na81-2.1, and D2/8-1 (lanes 1 to 5), and then separated by 10% SDS-PAGE. The wild-type NS1 and NS1' are marked with arrows, and the truncated proteins of p45 and p39 are positioned by asterisks.

tured prior to electrophoresis, the monomeric forms of NS1 and NS1' of normal sizes were both detected in primary infection of N18 (lane 2). In C2-2 cells, both p45 and p39, in addition to the normal NS1 and NS1', were detected (lane 4). In the absence of heat denaturation, all of the NS1 migrated as the homo- and heterodimeric forms (Fig. 3, lanes 3 and 5) (Lee *et al.*, 1991; Fan and Mason, 1990; Flamand *et al.*, 1992). These results clearly indicate that both p45 and p39 are the truncated forms of JEV NS1.

To further understand the structure of p45 and p39, we performed immunoprecipitation using five different anti-NS1 monoclonal antibodies (see Materials and Methods). Among them, four monoclonal antibodies, Na39-3.1, Na41-2, JE7/45-2, and Na81-2.1, have previously been shown to recognize conformational epitopes of NS1 although the precise epitopes have not been mapped (see Materials and Methods). The results showed that all four antibodies precipitated the wild-type NS1 and NS1' as well as p45 and p39 (Fig. 4B). It was unclear why p45 and p39 were more efficiently precipitated than NS1 and NS1' by these monoclonal antibodies (Fig. 4B). In contrast, monoclonal antibody D2/8-1, which has been characterized to recognize a linear epitope located at the amino acid positions 90 to 95 in the N-terminal one-third of JEV NS1 (Fig. 4A; see Materials and Methods), precipitated only the wild-type NS1 and NS1' and not the truncated NS1s (Fig. 4B, lane 5). This result strongly suggests that both p45 and p39 observed in C2-2 cells are truncated at their N-termini.

We next examined the glycosylation status of truncated

NS1s in C2-2 cells. Both NS1 and NS1' contain two predicted glycosylation sites at the amino acid positions 105 and 182 (Fig. 5A), and glycosylated moieties are about 6 kDa in mass. After complete digestion by endoglycosylase F (Endo F), the sizes of NS1 and NS1' decreased from 48 to 42 kDa and from 55 to 49 kDa, respectively, as expected (compare Fig. 5B, lane 1 with 4, and 2 with 5). Interestingly, both p45 and p39 also were susceptible to Endo F digestion: their sizes were reduced from 45 to 40 kDa and from 39 to 33 kDa, respectively, after Endo F digestion (Fig. 5B, compare lane 3 with 6). These results suggest that, in C2-2 cells, (i) the generation of the truncated NS1s did not interfere with the normal glycosylation of wild-type NS1 and NS1'; (ii) the truncated NS1s, like their wild-type counterparts, are translocated into ER and glycosylated; and (iii) the truncated proteins p45 and p39 retained both glycosylation sites. These results, combined with the failure of antibody D2/8-1 to recognize p45 and p39, suggest that these two proteins likely have truncations in their N-terminal 105th amino acid. Their possible relationships are indicated in Fig 5A: p45 might be the truncated form of NS1', whereas p39 might be the truncated form of NS1, both having an approximate 10-kDa deletion.

Detection of truncated NS1 in other persistently JEV-infected cells

To determine whether the truncation of NS1 and NS1' is a general characteristic of JEV persistent infection,

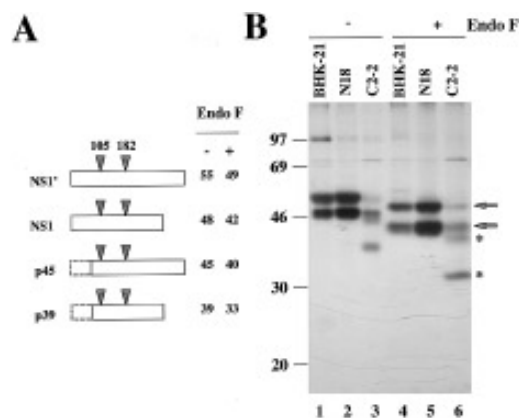


FIG. 5. The analysis of glycosylated modifications of NS1, NS1', and their truncated derivatives by Endo F digestion. (A) Schematic diagram of the NS1, NS1', and their truncated products shown in rectangles. The glycosylation sites are indicated by arrowheads on the top of the rectangles. The dashed lines at the N-termini of rectangles are designated as the predicted truncation regions of NS1 in C2-2 cells. The expected changes of molecular weights from precipitated NS1s treated either without (–) or with (+) Endo F digestion are numerically depicted at the right of each rectangle. (B) Cell lysates from JEV primary infection of BHK-21 (lanes 1 and 4) and N18 cells (lanes 2 and 5) and from persistent infection of C2-2 cells (lanes 3 and 6) were immunoprecipitated by anti-NS1 monoclonal antibodies and then were treated either without (lanes 1 to 3) or with (lanes 4 to 6) Endo F at 37° for 16 hr. The deglycosylated NS1 and NS1' (lanes 4 to 6) are indicated by arrows, and the deglycosylated truncated p45 and p39 (lane 6) are marked by asterisks.

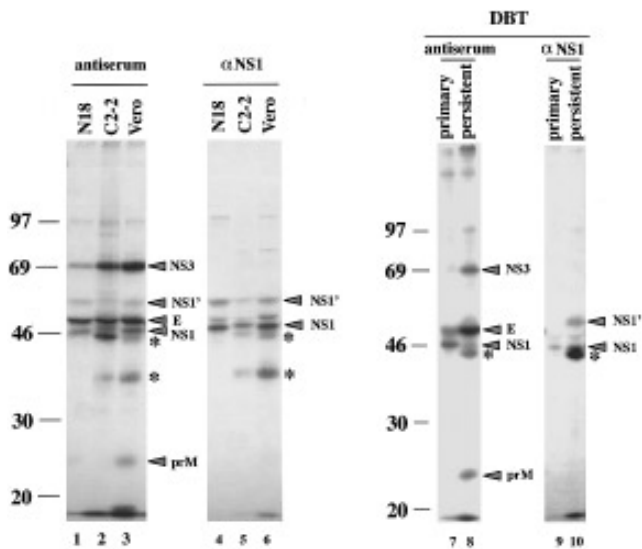


FIG. 6. Detection of truncated NS1 in several persistently infected cell lines. [³⁵S]Methionine-labeled lysates were immunoprecipitated with either polyclonal antiserum specific for JEV (antiserum, lanes 1 to 3 and 7 and 8) or with monoclonal antibodies specific for NS1 (α NS1, lanes 4 to 6 and 9 and 10). Cell lysates isolated from primary JEV infections comprise N18 (lanes 1 and 4) and DBT (lanes 7 and 9); lysates from persistent infections include C2-2 (lanes 2 and 5), Vero (lanes 3 and 6), and DBT (lanes 8 and 10). Immunoprecipitations were analyzed by SDS-10% PAGE. The truncated NS1s are indicated by asterisks.

we examined the NS1 protein in two other persistently infected cell lines established in Vero and DBT cells. The establishment of JEV persistency in Vero cells was similar to that in N18 cells; primary infection of JEV causes a severe CPE in Vero cells at 30 hr p.i., and >90% of the infected cells were lysed at 40 hr p.i. Surviving cells were grown into a cell population and showed a slight morphological change. The growth rate of these cells was about 30% slower than that of the uninfected Vero cells. JEV persistency in these Vero cells was verified by positive IFA staining of viral protein and continuous release of infectious virus for more than 3 months (data not shown). By contrast, the establishment of JEV persistency in DBT cells was significantly different from that in N18 and Vero cells. After infection, no obvious CPE was seen in DBT cells, although the infected cells grew about 30% slower than did uninfected DBT cells. During passages, such infected DBT cells were periodically checked for their viral antigen expression and virus release; about 90% of the cells were shown to have positive IFA staining in the cytoplasm (data not shown), and virus titers ranging from 10^4 to 10^5 were detected in culture media for more than 3 months. These results indicate that these cells were persistently infected with JEV.

NS1 expressed in persistently JEV-infected Vero and DBT cells was investigated by immunoprecipitation using virus-specific antiserum (see Materials and Methods) and anti-NS1 monoclonal antibodies (Fig. 6). When pre-

cipitated by antiserum, extra protein bands migrating faster than the normal NS1 were detected from all persistent infections of C2-2 (Fig. 6, lane 2), Vero (lane 3), and DBT cells (lane 8). These abnormal proteins were truncated NS1 proteins, as demonstrated by immunoprecipitation by monoclonal antibodies against NS1. In persistently infected Vero cells, two faster migrating proteins (Fig. 6, lane 6), which closely resembled the truncated NS1s p45 and p39, seen in C2-2 cell lysate, were detected (lane 5). As a control, primary infection of Vero cells did not express these faster migrating proteins (data not shown). In contrast, in persistently infected DBT cells only one species of faster migrating protein was detected (Fig. 6, lane 10). Again, this truncated NS1 protein was not detected in primary infection (Fig. 6, lane 9). Taken together, these results clearly indicate a strong relationship between abnormal NS1 expression and JEV persistency in cell cultures.

Analysis of the NS1-coding region on virus RNA isolated from persistent infection of N18 cells

To establish the origin and nature of the NS1 truncations in C2-2 cells, we carried out RT-PCR and direct sequencing to study the possible deletions and/or mutations in the viral genomic RNA in C2-2 cells. Total RNA purified from C2-2 cells was amplified by RT-PCR using primer pairs as described in Fig. 7A. The positions of primers chosen were based on the assumption that the truncated NS1s should still contain the signal peptides at their N-termini (Flamand *et al.*, 1992) in order to be translocated into the ER for proper glycosylation. As demonstrated in Fig. 7B, both C2-2 cells and primary infection of N18 cells yielded PCR products of identical sizes; no truncated PCR products were detected in C2-2 cells (Fig. 7B, lanes 2 to 5). To detect mutations, we directly sequenced the RT-PCR products purified from agarose gel in Fig. 7B, lanes 4 and 5. Primer d (Fig. 7A) was used here as a sequencing primer to analyze the sequences around the junction of the predicted truncation site on NS1. The results demonstrated that no mutation was found in the PCR products from persistent infection of C2-2 cells, compared to that from primary infection of N18 cells (data not shown). Moreover, the sequence result of PCR products purified from Fig. 7B, lanes 2 and 3, which cover the entire NS1-coding region, revealed that no mutation was found in NS1 from C2-2 cells compared to that from primary infection of N18 cells (data not shown). Thus, these results suggest that the truncations of NS1 and NS1' seen in persistent infection of C2-2 cells did not result from the mutations of the NS1 gene in the viral RNA, but most likely resulted from posttranslational cleavage caused by defects in other genes.

DISCUSSION

We have successfully established persistent JEV infection in murine neuroblastoma cells (N18), murine astrocy-

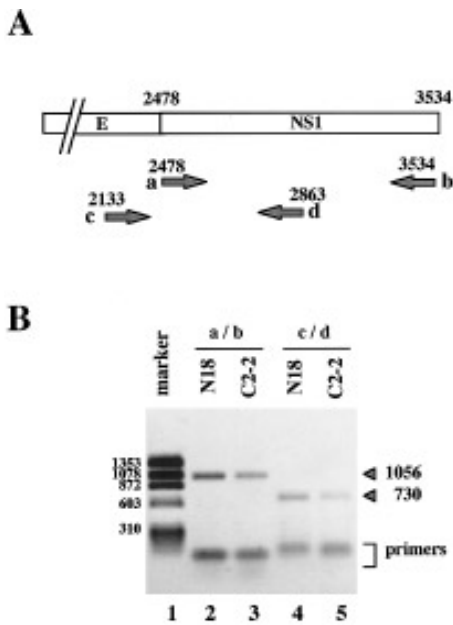


FIG. 7. Analysis by RT-PCR and direct sequencing of the NS1 coding regions on viral RNAs isolated from JEV primary infection of N18 cells and from persistent infection of C2-2 cells. (A) Schematic diagram of the primers used in RT-PCR analysis. The locations and polarities of each primer are shown by numbers and arrows, respectively. The primers a and c are in the same polarity as virus genomic RNA, whereas the primers b and d are complementary to the virus genomic RNA. The locations of primers a and c start from their 5'-end corresponding to the nucleotide positions 2478 and 2133 on the virus genome, respectively; primers b and d start at 3534 and 2863, respectively. (B) RT-PCR products analyzed by agarose gel electrophoresis. Total RNA, isolated from primary infection of N18 cells and from persistent infection of C2-2 cells, was subjected to RT-PCR using primer pairs of a and b (lanes 2 and 3) and of c and d (lanes 4 and 5) as shown in A. PCR products were assayed by gel electrophoresis and stained with ethidium bromide. Markers in lane 1 are ϕ X174-*Hae*III fragments. The predicted lengths of RT-PCR products are indicated by arrowheads accompanied by numbers in nucleotides. The resulting frame is shown in its negative.

toma cells (DBT), and Vero cells. In particular, the establishment of viral persistency in the C2-2 cell clone, derived from the persistently infected N18 cells, provides an important system for studying the persistent JEV infection in neuronal cells. The main finding of this study is the detection of truncated NS1s which were tightly associated with JEV persistency in various cell lines. We have recently also observed an aberrant NS1 expression in the persistent JEV infection of NT2 cells, a human neuronal progenitor cell line (unpublished observation). The generality of this abnormal NS1 expression pattern associated with all of the persistently infected cells studied so far strongly suggests that NS1 plays a crucial role in JEV persistency. Thus, the aberrant NS1 expression pattern may be used as a molecular marker for the identification of JEV persistency.

The truncation of NS1s in C2-2 cells likely resides in their N-terminal 105th amino acid, as evidenced by (i)

the failure of antibody D2/8-1 to recognize p45 and p39 (Fig. 4) and (ii) the possession of normal glycosylated moieties of the truncated p45 and p39, similar to that of their counterpart wild-type NS1s (Fig. 5). However, our data indicated that no mutation was detected in the NS1 region of the viral genome in C2-2 cells, suggesting that the truncations of NS1 and NS1' seen in C2-2 cells did not result from the mutations in the NS1 gene, but most likely resulted from posttranslational cleavage caused by defects in other genes. It is necessary in the future to examine whether there is mutation in other viral genes leading to aberrant expression of NS1 in C2-2 cells.

The biosynthesis of flavivirus NS1 has been demonstrated to utilize the cellular secretory pathway, i.e., ER and Golgi complex (Mason, 1989; Fan and Mason, 1990; Blitvich *et al.*, 1995). Conceivably, the truncation of NS1 in C2-2 cells was a result of an aberrant proteolytic cleavage, which occurred within ER or Golgi apparatus after the translocation of NS1. The protease responsible for this cleavage is unlikely to be the known viral protease NS3, which is a trypsin-like serine protease responsible for the cleavages of NS2A-2B, NS2B-3, NS3-4A, and NS4B-5, since its cellular location is mainly in the cytoplasm (reviewed in Chambers *et al.*, 1990). A cellular protease or an unknown viral protease present in ER or Golgi apparatus of C2-2 cells is likely involved in this cleavage event. The coexistence of the wild-type and the truncated NS1s in all JEV-persistent cells (Figs. 3 and 7) indicated that this aberrant proteolytic cleavage was incomplete, suggesting that the activity of the putative protease is tightly regulated. We have failed to identify the cause of abnormal cleavage; nonetheless, we have established the association between NS1 truncation and JEV persistency in various culture systems.

Whether the generation of truncated NS1s is a cause or an effect of JEV persistency in cell cultures remains unclear. Nonetheless, our data do indicate that the expression of truncated NS1s is a dominant phenotype (Fig. 2) associated with the maintenance of JEV persistency in a variety of cell lines. The establishment of persistent infection is a dynamic process as a result of interaction between virus and host. The NS1 truncation could be a result of change of host cells, altering the specificity of viral or cellular proteases. Conceivably, JEV persistency in C2-2 cell requires the relative ratio between the wild-type and the truncated NS1s to regulate the balance between lytic infection and temperate persistency, presumably through interfering with the process of viral assembly when nucleocapsid buds into the lumen of the ER (reviewed in Chambers *et al.*, 1990). This hypothesis is supported in part by our observation that both the wild-type and the truncated NS1s were detected in all of the persistently infected cell lines (Fig. 6). In addition, flaviviral NS1 protein has been suggested to play a role in the process of virion assembly or maturation within the cellular secretory pathway (Mason, 1989; Fan and Ma-

son, 1990). This interpretation is also consistent with the finding that all of the cells showed positive viral antigen staining and yet only 1% of cells released viruses in the infectious cell center, and the culture produced very low virus titers (Table 1).

The shift from cytolytic infection to persistent infection in a susceptible cell must be occurring under strong selective pressures on both the virus and the host. As suggested for reovirus persistency (Ahmed *et al.*, 1981), in order for virus to persist in cell culture, on one hand the cell should adjust to sustain JEV replication without being lysed; on the other hand, JEV has to change so that it cannot damage the host cell. In agreement with this hypothesis, the observation that the four antigen-negative cell clones (Table 1) showed reductions of the virus production when superinfected with JEV suggests that they may be the relics of mutated cells selected during infection process. Furthermore, the virus released from the persistently infected C2-2 cells did not produce persistent infection or cause abnormal NS1 processing upon primary infection of N18 cells, suggesting that the virus itself is not sufficient to establish persistent infection. Moreover, the antigen-positive clone C2-2 likely represents a successful endpoint of the mutation course, which survives simply because it properly balances the dilemma between the cytolytic and the persistent infection.

In other virus systems, the altered viral gene products have been shown to be responsible for viral persistent infections. For instance, the mutations in capsid proteins VP1 and VP2 of poliovirus have been shown to be required for the virus to persistently infect HEp-2 cells (Calvez *et al.*, 1993); the mutation and the restricted expression of M gene has been linked to the persistent measles virus infection in human brain (Cattaneo *et al.*, 1988; Baczkó *et al.*, 1993); the His-55 in glycoprotein E2 of Sindbis virus is an important neuroadaptive mutation that allows avirulent persistent infection to become neurovirulent (Levine and Griffin, 1993); a single amino acid change in viral glycoprotein markedly increased the ability of lymphocytic choriomeningitis virus to persist in adult mice (Matloubian *et al.*, 1990). Here, we provide an additional example of this kind, i.e., the truncated NS1s were tightly correlated with the persistent JEV infection in cell cultures. However, in contrast to other viruses, the viral gene products associated with JEV persistency are viral nonstructural proteins.

In conclusion, (i) JEV persistency has been established in several immortalized cell lines including neuroblastoma N18 cells, astrocytoma DBT cells, and Vero cells; (ii) C2-2 clone derived from N18 cells expressed truncated NS1s in addition to normal ones, and the truncation of abnormal NS1s was localized to their N-termini; (iii) truncated NS1s were also observed in persistently infected Vero and DBT cells, but not in their primary infection counterparts, indicating a strong association

between abnormal NS1 expression and JEV persistency; (iv) the truncation of NS1 in C2-2 cells probably resulted from an additional posttranslational proteolytic cleavage occurring in the persistently infected cells; and (v) these results suggest a probable involvement of dysfunctional NS1 in the establishment and/or maintenance of JEV persistency in culture systems.

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